An NIH Director's Wednesday Afternoon Lecture Series Event
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A Day-long Minisymposium

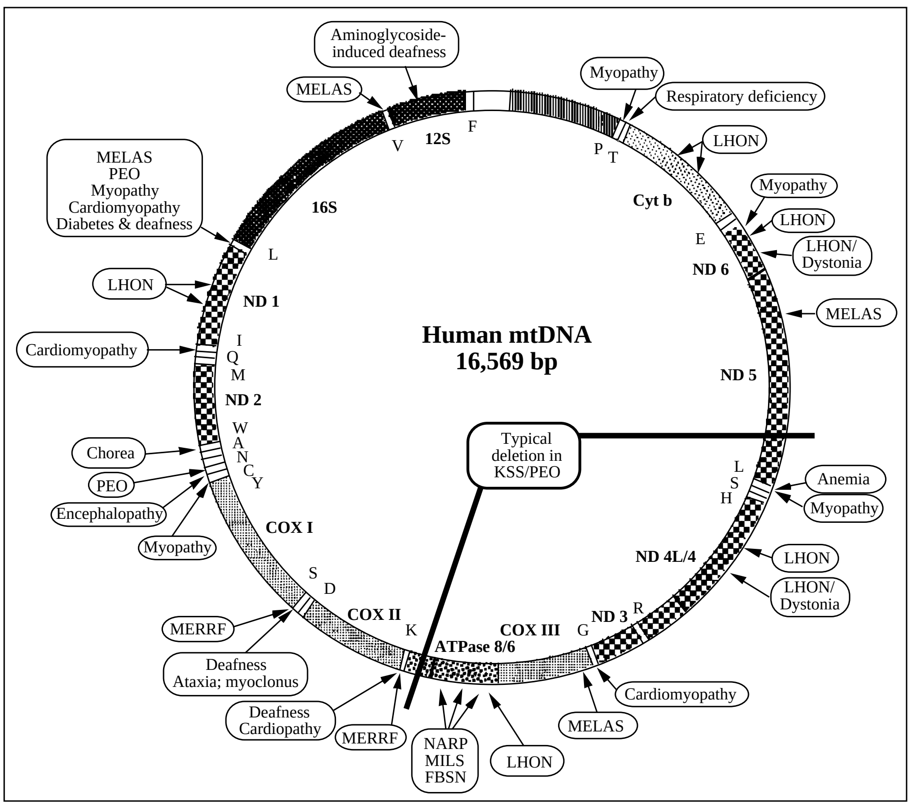
Mitochondria: Genetics, Health, and Disease
2 December 1998
Featuring

The Wednesday Afternoon Lecture
by

Dr. Eric A. Schon (Columbia)

Molecular Genetics of Human Mitochondrial Disease

Morbidity map of the human mitochondrial genome
1997



FBSN: familial bilateral striatal necrosis
KSS: Kearns-Sayre syndrome (sporadic)
LHON: Leber's hereditary optic neuropathy
MELAS: mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes
MERRF: myoclonus epilepsy with ragged-red fibers
MILS: maternally-inherited Leigh syndrome
NARP: neuropathy, ataxia, and retinitis pigmentosa
PEO: progressive external ophthalmoplegia

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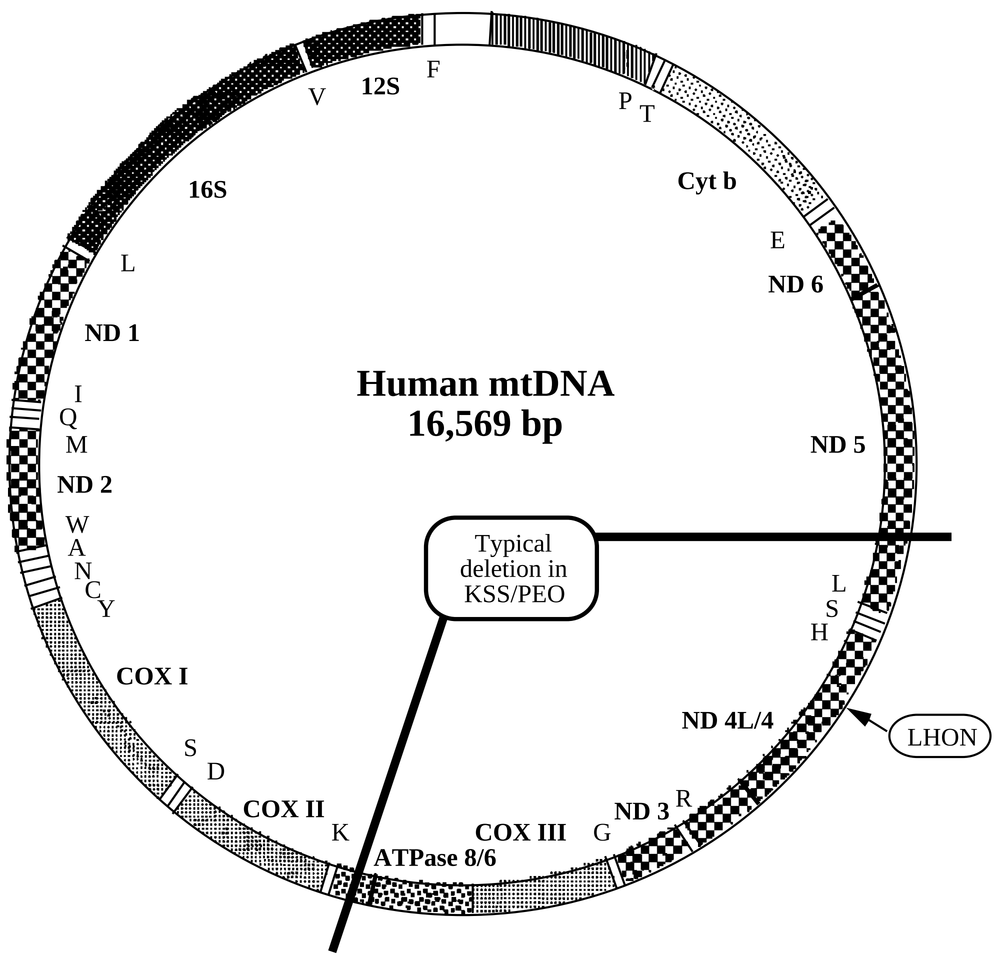
Morbidity map of the human mitochondrial genome 1988



MITOCHONDRIA: GENETICS, HEALTH, AND DISEASE
MINISYMPOSIUM 2 DECEMBER 1998

Lectures: Masur Auditorium, Clinical Center, NIH

Posters/Exhibitors: Visitor Information Center, Clinical Center, NIH

- 0745 Registration, Poster Set-up, Continental Breakfast in Exhibit Area
- 0830 Dr. David A. Clayton (HHMI): Mitochondrial DNA Control Features
- 0905 Dr. William C. Copeland (NIEHS): Avoidance of Mitochondrial DNA Mutations by DNA Polymerase Gamma
- 0940 Dr. Vilhelm A. Bohr (NIA): Oxidative DNA Damage Repair in Mammalian Mitochondria
- 1015 Poster Session/Coffee Break in Product Exhibit Area
- 1045 Dr. Wing Hang Tong (NICHD): Abnormalities of Mitochondrial Iron Metabolism and Human Disease
- 1120 Dr. Steven J. Zullo (NIMH): *In situ* Localization of the common Human 4977bp Mitochondrial DNA Deletion Mutation
- 1200 Lunch Break
- 1330 Dr. Mariana Gerschenson (NCI): Mitochondrial Genotoxic and Functional Consequences of Chemotherapeutic Drugs
- 1400 Poster Session/Coffee Break in Product Exhibit Area
- **1500 Wednesday Afternoon Lecture: Dr. Eric A. Schon (Columbia):Molecular Genetics of Human Mitochondrial Disease**
- 1600 Reception/Poster Session in Product Exhibit Area
- 1700 Poster Session/Product Exhibition Closes

Continental Breakfast, Coffee Breaks, Lunch Break, and Reception sponsored by the Technical Sales Association

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Colombini, Marco	The permeability of the mitochondrial outer membrane: influence of different VDAC isoforms, BAX, and BCL-XL
Fernandez-Salas, Ester	p53 and TNF-a regulate a mitochondrial chloride channel protein associated with apoptosis in keratinocytes
Kinnally, Kathleen W	INVESTIGATIONS INTO THE ROLE OF MITOCHONDRIA AND THEIR PROTEIN TRANSLOCATING CHANNELS IN APOPTOSIS.
Kowaltowski, Alicia J.	Bcl-2 prevents mitochondrial permeability transition due to increased mitochondrial reducing power
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Longley, Matthew J.	Identification of 5'-deoxyribose phosphate lyase activity in human DNA polymerase gamma and its role in mitochondrial base excision repair in vitro
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Eells, Janis T	EVIDENCE FOR MITOCHONDRIAL DYSFUNCTION IN TOXIC AND NUTRITIONAL RETINOPATHIES AND OPTIC NEUROPATHIES
Gropman, Andrea	Pediatric dystonia due to mtDNA mutation at np 14459--report of the fourth family
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Levin, Barbara C.	HETEROPLASMY RESPONSIBLE FOR DIFFICULTY EXPERIENCED IN SEQUENCING THE HUMAN MITOCHONDRIAL DNA HV1 REGION CONTAINING THE C-STRETCH
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Dani, Sergio U.	The $mtDNA^{4977}$ deletion, a tumor suppressor mutation
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Melnov, Sergei B.	IN SITU PCR ASSAY FOR “COMMON” HUMAN MITOCHONDRIAL DNA DELETION: METHODOLOGICAL AND PRACTICAL APPROACHES
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Stierum, Rob	PURIFICATION AND CHARACTERIZATION OF A RAT LIVER MITOCHONDRIAL THYMINE GLYCOL ENDONUCLEASE
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Diagnostic and Treatment Modalities	
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CLAYTON

MITOCHONDRIAL DNA CONTROL FEATURES

David A. Clayton
Howard Hughes Medical Institute; 4000 Jones Bridge Road; Chevy Chase, Maryland 20815-6789

The discovery of human disease-causing mitochondrial DNA (mtDNA) mutations raised possibilities for understanding the pathogenesis of mitochondrial disorders. With the application of contemporary molecular genetics, it has been shown that mtDNA mutations are an important cause of mitochondrial disorders and specific mtDNA mutations have been found to be the underlying basis of previously defined clinical syndromes. Mutations of mtDNA have also been associated with aging and neurodegenerative conditions. This talk will address the issue of mtDNA replication and expression principally from the standpoint of key nuclear gene products involved in these events, and the mtDNA regions of regulation

Replication of mtDNA

Replication of human mtDNA is under relaxed control; this means that although the population of mtDNA molecules doubles with every cell doubling, there is no mechanism to identify each and every molecule for one replication event. Thus, some molecules replicate twice (or perhaps more) and others not at all. Consistent with this is the apparent lack of restriction on mtDNA replication with regard to cell cycle phase.

Replication of mtDNA involves mtDNA polymerase and additional factors. Human mtDNA has two distinct and separate origins of replication. The origin of heavy (H)-strand (leading-strand) replication, $O_{H,}$ is located in the displacement-loop (D-loop) region, while the origin of light (L)-strand (lagging-strand) replication, O_L, is contained within a cluster of five tRNA genes. The commitment to replicate mtDNA initiates at O_H; this requires both RNA synthesis and subsequent processing events to prime DNA synthesis. When the leading strand has elongated to two-thirds of its total length, O_L is exposed and sponsors initiation of lagging-strand replication.

The replication of mtDNA is special in that it requires first transcription by mitochondrial RNA (mtRNA) polymerase, followed by discrete processing of transcripts to form proper primers for subsequent elongation by mtDNA polymerase. Other proteins, such as single-strand DNA binding protein (SSB) and transcription factors, are likely or known to be required.

Transcription of mtDNA

The well known displacement-loop (D-loop) region is the control site for both transcription and replication of mtDNA. Each strand of mtDNA contains a major dedicated promoter; i.e. the H-strand promoter (HSP) and the L-strand promoter (LSP). The LSP serves two purposes; it is the promoter for one protein and eight tRNA genes, and transcripts initiated at the LSP are used as primers for leading-strand mtDNA replication. The transitions from RNA to DNA synthesis occur over a region of short conserved sequences termed conserved sequence blocks (CSBs) I-III.

Transcription of mtDNA requires mtRNA polymerase, mitochondrial transcription factor A (h-mtTFA), and likely some additional factors. The h-mtTFA protein binds at the upstream region of both the HSP and LSP and most likely activates transcription as a result of DNA binding. It is known that h-mtTFA has the capacity to unwind and bend DNA, thereby suggesting the manner in which transcription initiation takes place.

Transcripts initiated from the HSP are present in different relative amounts. The 12S and 16S ribosomal RNAs are 50-100 times more abundant than the messenger RNAs. The high production of rRNAs is probably due, at least in part, to transcription termination, since general studies have demonstrated that a tridecamer sequence embedded in the $tRNA^{Leu(UUR)}$ gene is bound by a 34-kDa protein (mtTERM); this protein/DNA complex sponsors transcription termination.

Nuclear Gene Products in Mitochondrial Disease

Key proteins (and their genes) involved in the expression and replication of mtDNA have now been identified. This allows investigators to test for mutations in, for example, mtTFA, mtRNA polymerase, mtDNA polymerase, mitochondrial SSB, and other nuclear genes that will certainly become available in the near future. The target sites of interaction of these activities on mtDNA are known with reasonable precision. Therefore, mitochondrial diseases resulting from failure-to-function at the level of mtDNA expression and replication can and should be addressed by both analysis of mtDNA target sequences in regulatory regions and the nature and expression of the nucleus-encoded activities that interact with them.

COPELAND

Avoidance of Mitochondrial DNA Mutations by DNA Polymerase Gamma

William C. Copeland
Laboratory of Molecular Genetics, National Institute for
Environmental Health Science, Research Triangle Park, NC 27709

Human mitochondrial DNA evolves faster and is more prone to oxidative damage than is nuclear DNA. Point mutations and deletions in this mitochondrial genome give rise to a wide range of mitochondrial dysfunctional diseases affecting some 50 million people in the US. These mutations may occur during replication by the DNA polymerase gamma. The DNA polymerase gamma differs from the nuclear DNA polymerases due to its sensitivity to antiviral nucleotide analogs, such as AZT and dideoxynucleotides. These analogs cause mitochondrial toxicity by inhibiting the DNA polymerase gamma. To understand the role of the DNA polymerase gamma in this mutagenesis process and its sensitivity to antiviral compounds we have cloned the DNA polymerase gamma genes and cDNA from S. pombe, D. melanogaster and Homo Sapiens. The recombinant human mitochondrial DNA polymerase gamma protein has been functionally overexpressed greater than 100 fold in insect cells by a recombinant baculovirus. The recombinant pol gamma and two mutant derivatives have been purified to homogeneity and enzymatically characterized. By changing a Tyr951 to Phe in the DNA polymerase gamma we have rendered the enzyme 5000 times more resistant to dideoxynucleotides with only a minor effect on polymerase activity. Using an exonuclease deficient mutant we are assessing the contribution of proofreading and nucleotide selection in replication fidelity. We have discovered a new activity, a 5'-deoxyribose 2'-phosphatase activity, intrinsic to the catalytic subunit of human DNA polymerase gamma. This dRP lyase activity functions in mitochondrial base excision repair in vitro. Thus, DNA polymerase gamma participates in both DNA replication and mitochondrial DNA repair.

BOHR

Oxidative DNA Damage Repair in Mammalian Mitochondria

Vilhelm A. Bohr
Chief, Laboratory of Molecular Genetics National Institutes on Aging, NIH.

1. There is DNA repair activity in mitochondria.
2. DNA repair of oxidative DNA damage is efficient in mammalian mitochondrial DNA
3. Some DNA lesions are repaired and others not in mammalian mitochondria, suggesting that some but not all of the DNA repair pathways present in nuclear DNA is present in mitochondria.
4. Oxidative DNA damage of some types accumulate in mitochondrial DNA with age.
5. DNA repair capacity of oxidative DNA damage in mitochondria does not appear to decline with age.
6. It is a great challenge biochemically and from a aging theory standpoint to investigate the mitochondrial DNA damage processing

TONG

Abnormalities of Mitochondrial Iron Metabolism and Human Disease

Wing-Hang Tong
National Institute of Child Health and Human Development; Section on Human Iron Metabolism; Cell Biology and Metabolism Branch.

Characterization of a Multiprotein Complex involved in the Biosynthesis of Iron-Sulfur Proteins in MitochondriaIron-sulfur (Fe-S) proteins are essential components of respiratory chain complexes and citric acid cycle, and recent studies on mitochondrial diseases have indicated that these metalloproteins are critical targets for oxidative damage. Recent studies in Friedreich's ataxia patients have shown deficiency in mitochondrial Fe-S cluster dependent enzymes, presumably as a result of mitochondrial iron overload and increased oxidative stress. The purpose of the present study is to elucidate the biochemical mechanism of Fe-S cluster assembly and disassembly in vivo, and to understand the cell biology of metalloprotein metabolism in the context of compartmentalized assembly. Studies of metalloprotein assembly in bacteria indicated that NifU and NifS proteins are required for biosynthesis of Fe-S proteins in these organisms. We have cloned the human homologues of NifU and NifS, and examined their functions, expression profiles, subcellular localizations, and regulations by environmental variables. Sequence analysis and preliminary biochemical studies indicate that NifS is a cysteine desulfurase while NifU contains a single iron binding site. Immunoprecipitation and immunofluoresence experiments show that different isoforms of NifU and NifS localize to the mitochondria, the cytosol and the nucleus, and the mitochondrial isoforms of NifU and NifS are associated in a multiprotein complex. Taken together, these results suggest that NifS may serve to sequester inorganic sulfur while NifU may serve as a iron carrier for biosynthesis of Fe-S proteins in the mitochondria. Furthermore, we have shown that these proteins are differentially regulated by environmental and developmental signals which would allow tissues to adjust their energy production and iron metabolism according to energy demands and oxidative stress. Primary defects in mitochondrial function are implicated in over 100 diseases and iron-catalyzed Fenton chemistry in the mitochondria is thought to contribute to aging and age-related degenerative diseases including Parkinson disease, Alzheimer's disease and cardiomyopathies. Increased understanding of mitochondrial iron metabolism will lead to greater understanding of the molecular basis of mitochondrial disorders and development of new therapeutic strategies.

In situ PCR Localization of the "Common" Human Mitochondrial DNA Deletion

Steven J. Zullo[1], Sergei Melnov[2,3], William F. Blakely[3], Carl R. Merril[1]
[1]Laboratory of Biochemical Genetics, National Institute of Mental Health-NIH, Bethesda, MD USA; [2]Institute of Nuclear Medicine and Endocrinology, Minsk, Belarus; [3]Armed Forces Radiobiology Research Institute, Bethesda, MD USA

It was noted at a recent international conference on Alzheimer's disease in Cochin, India, that five percent of the world's elderly population, 18 million victims, suffers from Alzheimer's disease. By 2020 the number is expected to climb to 30 million, 75 percent in developing countries in Asia and Latin America. Mitochondrial dysfunction could play an important role in Alzheimer's disease, irrespective of suspected or known disease genes. Indeed, incidence of the human 4977 bp mitochondrial DNA deletion (mtDNA^{4977} deletion) has been measured at increased levels in Alzheimer's disease, conditions associated with chronic hypoxia, and radiation exposure, as well as chronic progressive external ophthalmoplegia (CPEO), Kearns/Sayre syndrome, and Pearson's marrow syndrome. Metabolically detrimental effects of the deletion reported in muscle and multi-organ system diseases are not detected until the deletion level reaches 50%-60%, based on *in vitro* assays. However, the incidence of the deletion associated with dysfunction in the central nervous system (CNS) tissue, is routinely around 1% or less (*e.g.*, in Alzheimer's disease and conditions associated with chronic hypoxia). In muscle tissue and cell culture, the deleted mtDNA population is such a large fraction of the total mtDNA that *in situ* hybridization assays identifying both deleted and undeleted mtDNA have localized the deletions to the abnormal "ragged red fibers" of the diseased tissue. In CNS tissue however, where neurons are highly dependent on mitochondrial oxidative phosphorylation, these deletions may be present at a low level in all cells in the sampled tissue, or they may be concentrated in only a few cells. Thus, quantitative assays for this deletion are needed at the cellular level to determine the distribution of the deletion, and to study the possible effects of the deletions. The current methods utilized to detect the deletion are not sufficient to localize the deletion when it is present at such low levels as found in CNS tissue. Likewise, a recent study has recorded that the mtDNA^{4977} deletion is induced by radiation depending on the sensitivity of the cell type. Thus a reliable assay could also be useful for radiation dose assessment. We have developed an *in situ* polymerase chain reaction (PCR) assay that can detect the deletion within discrete cellular regions. We have utilized established lymphocyte cell lines, one that maintains the mtDNA^{4977} deletion incidence at around 50% of the total mtDNA, and two containing little or no deleted mtDNA. The cell lines were derived from a male with Pearson's marrow syndrome, his mother with CPEO, and his asymptomatic sister, respectively. We observed that this mtDNA deletion is associated with apoptotic changes in the cultured lymphocytes. The distribution of the mtDNA^{4977} deletion in the cultured lymphocytes ranges from isolated cellular regions to cells that are apparently saturated with mtDNA^{4977} deletions. These observations are consistent with a progression of the deletion from a single locus to deletions throughout the cell. These studies could provide important insights into the mitochondrial involvement in apoptosis and pathophysiological processes in disorders such as Alzheimer's disease. The *in situ* PCR mtDNA^{4977} deletion assay we have developed could be directly applicable for radiation dose assessment of lymphocytes from blood samples.

GERSCHENSON

Mitochondrial Genotoxic and Functional Consequences of Chemotherapeutic Drugs.

M. Gerschenson
Carcinogen-DNA Interactions Section, LCCTP, NCI, NIH, Bethesda, MD.

Chemotherapeutics drugs which are incorporated or bind to DNA are used to treat cancer and AIDs. Unfortunately one of the consequences of this type of therapy is the potentially toxic effects to the mitochondrial genome. Currently, we are investigating the end results of transplacental exposure of a pyrimidine nucleoside, zidovidine (AZT), in a monkey animal model.

Chronic AZT exposure causes depletion of mitochondrial DNA (mtDNA), abnormal oxidative phosphorylation and morphological changes in skeletal muscle in 5-25% of HIV-1 positive humans. Furthermore, AZT is given to most HIV-1 positive pregnant women in the US for the last six months of pregnancy with unknown fetal mitochondrial consequences. AZT incorporation into mtDNA has been shown in fetal patas monkey tissues after transplacental AZT exposure(Olivero, O. A., JNCI, Vol. 89, pp. 1602-8, 1997). To model the human exposure, pregnant Erythrocebus patas monkeys were given a daily dose of AZT equivalent to 20% and 80% of the human dose for the second half of gestation. Electron microscopy of the AZT exposed tissues demonstrated dose-related abnormal, degenerated, enlarged, vacuous mitochondria with decreased cristae. NADH dehydrogenase (Complex I) specific activity was also decreased in both tissues in a dose dependent manner in AZT-exposed animals. Succinate dehydrogenase (Complex II) activity was increased in the exposed skeletal muscle compared to the controls. Cytochrome c oxidase (Complex IV) activity was decreased 2-10 fold in skeletal muscle and increased 2-10 fold in the heart of fetal monkeys exposed to 1.5 mg AZT/kg body weight, and increased 2-10 fold in both tissues from fetal monkeys exposed to 6.0 mg AZT/kg body weight. It is possible that the observed alterations are due to early termination of mtDNA replication caused by the incorporation of AZT. This is consistent with the lack of cristae in the mitochondria and the decrease in Complex I activity, which has seven polypeptides encoded by the mt genome. These results demonstrate that the transplacental administration of AZT to pregnant patas monkeys results in significant changes in fetal mitochondria at doses similar to those given to pregnant women, suggesting that children of HIV-1 positive pregnant women given AZT should be followed for potential mitochondrial myopathies.

Abstracts in order of Poster Number

Apoptosis and Mitochondria

Poster 1

TISSUE-SPECIFIC PATHWAYS OF CYTOCHROME C RELEASE FROM MITOCHNDRIA.

Alexander Y. Andreyev and Gary Fiskum
Department of Anesthesiology
University of Maryland School of Medicine. MSTF Bldg., Rm. 534 10 South Pine Street
Baltimore, MD 21201 (aandreyev@anesthlab.ab.umd.edu, 410-706-2990, [FAX]: -2550)

Release of cytochrome c (CytC) from mitochondria into the cytosol which triggers the apoptotic cascade in various cell death paradigms is widely attributed to the onset of $Ca2^{+}$-dependent, cyclosporin A (CsA)-sensitive mitochondrial permeability transition (MPT). Comparing isolated brain and liver mitochondria accumulating $Ca2^{+}$ in saline medium in the presence or absence of ATP and $Mg2^{+}$ we have observed a profound tissue-specific difference in the association of the two processes. In liver mitochondria under a variety of conditions CytC release strictly correlates with the extent of MPT-induced swelling and reaches 100% upon complete permeabilization. CsA, alone or in the combination with the other MPT inhibitors, suppresses the CytC release in accordance with the degree of inhibition of swelling. In contrast to such a strict interrelation in brain mitochondria CytC release proves to occur independently on the MPT. Both in the presence and in the absence of ATP and Mg2+ CytC release from brain mitochondria is limited to maximal 40% of total enzyme content and is insensitive to CsA. In the presence of ATP and Mg2+ the CytC release is not associated with any degree of mitochondrial swelling, as well as loss of Ca2+ uptake capacity or complete mitochondrial depolarization which are indicatives of the MPT. The observed moderate decrease in Dy is completely reversible upon Ca2+ sequestering by EGTA+Ca2+ ionophore A23187 but not by EGTA alone and therefore is not MPT-mediated. Ruthenium Red preventing Ca2+ uptake inhibits the CytC release but this inhibition can be abolished by uncoupler (DNP)-induced Dy drop not associated with swelling. In the absence of ATP and Mg2+ brain mitochondria are capable of the MPT manifesting itself in CsA-sensitive large amplitude swelling , glutathione release and loss of Ca2+ uptake capacity. All these changes in brain mitochondria are partial comparing to the effect of artificial pore-forming agent Alamethicin which causes complete MPT and 100% CytC release both in brain and liver mitochondria. (Supported by NIH NS34152 and by Bayer Corporation).

Poster 2

The permeability of the mitochondrial outer membrane: influence of different VDAC isoforms, BAX, and BCL-XL

Xiaofeng Xu and Marco Colombini
Dept. Biology, University of Maryland, College Park, MD 20742 (COLOMBINI@ZOOL.UMD.EDU, 301-405-6925, FAX –9358)

The permeability of the mitochondrial outer membrane can be quantitated by measuring the reduction in rates of mitochondrial processes produced by the presence of an intact outer membrane and processing these results with an appropriate mathematical description (Lee and Colombini, Methods in Cell Science 16: 71-81, 1997). For yeast (S. cerevisiae) mitochondria, the high intermembrane space NADH dehydrogenase activity allows convenient measurement of the outer membrane permeability to NADH. Mutants lacking the major pathway through the outer membrane, VDAC1, are particularly useful in that other proteins targeted to the outer membrane can be expressed and tested. Thus, in collaboration with M.J.Sampson and W.J.Craigen, each of the 3 mouse VDAC isoforms was individually expressed and each conferred a different level of permeability to the outer membrane indicating functional specialization of each isoform. This is consistent with the electrophysiological properties of these isoforms. The expression in this system (in collaboration with A.Gross and S.J.Korsmeyer) of reported channel formers located in the outer membrane, BAX and BCL-XL, revealed that only BAX increases the outer membrane permeability to NADH. Indeed, when BAX was expressed, the outer membrane did not limit NADH flux. This could be interpreted as BAX disrupting the outer membrane.

Poster 3

p53 and TNF-alpha regulate a mitochondrial chloride channel protein associated with apoptosis in keratinocytes.

Fernández-Salas, E., Sagar, M., Cheng, C., Weinberg, W. C., and Yuspa, S. H.
Laboratory of Cellular Carcinogenesis and Tumor Promotion, NCI; and Oral and Pharyngeal Cancer Branch, NIDCR; NIH, Bethesda, MD 20892 (esterf@nih.gov, 301-496-3248, [FAX]: 301-496-8709)

A novel chloride channel gene, clone mc3s5, has been identified from differential display analysis of differentiating mouse keratinocytes from p53(+/+) and (-/-) mice. The 4.2 kb cDNA contains an open reading frame of 762 bp encoding a 253 aa protein with 2 transmembrane domains. Mc3s5 protein shares extensive homology with the bovine vesicle chloride channel p64, the human chloride channels NCC27 and CLIC2, and the rat endoplasmic reticulum chloride channel p64H1. Mc3s5 mRNA is expressed to the greatest extend in heart, lung, liver, kidney, and skin with levels 2 to 5-fold higher in p53(+/+) mice compared with p53(-/-) mice. Basal expression of mc3s5 mRNA is 5-fold higher in p53(+/+) compared to (-/-) mouse keratinocytes in culture and is induced 3-fold in both genotypes following differentiation. Overexpression of p53 using an adenovirus encoding the wild-type protein induces mc3s5 mRNA 3-fold in the p53(+/+) and 6-fold in the p53(-/-) keratinocytes. In cultured keratinocytes, exogenous hrTNF-alpha up-regulates mc3s5 mRNA 3.5-fold at 6 h and mc3s5 protein 2-fold after 24 hrs of treatment. By cell fractionation both the fusion protein mc3s5-GFP and the internal mc3s5 are localized to the cytoplasm and mitochondria. Sucrose purified mitochondria from rat liver confirmed this localization pattern. Overexpression of mc3s5 in keratinocytes leads to cell death that can be inhibited by the caspase inhibitor Z-VAD, indicating activation of an apoptotic pathway. These data suggest that mc3s5 may participate in apoptotic pathways regulated by both TNF-alpha and p53.

Poster 4

INVESTIGATIONS INTO THE ROLE OF MITOCHONDRIA AND THEIR PROTEIN TRANSLOCATING CHANNELS IN APOPTOSIS.

R.C. Murphy[1, 2], A. Khodjakov[1], A. Moodie[1], E. Schneider[1], C. Rieder[1], C.A. Mannella[1], M. L. Campo[3], and K.W. Kinnally[1]

[1]Div. of Mole. Med., Wadsworth Center, and Dept. of Biomed. Sci., Univ. Albany, Albany, NY. Biology; [2]R.P.I., Troy, NY; [3]Dpto. de Bioq. Univ. de Extremadura, Spain.

Several laboratories have produced evidence that the commitment step of apoptosis is linked to the release of cytochrome c from mitochondria into the cytosol. Laser microsurgery was used to directly lyse selected numbers of mitochondria (thus releasing their components) in living cells which were then followed by video LM. The possible role in apoptosis of the protein import channel, MCC, of the mitochondrial inner membrane was also studied. A time line for the onset of apoptotic markers was established for human breast cells (MDA231) treated with teniposide. By patch-clamping mitochondria isolated from cells at various times after treatment, the conductance through the cyclosporin A sensitive-channel MCC was found to open early in apoptosis. Other early events included mitochondrial depolarization (JC-1 fluorescence) and loss of lipid asymmetry in the plasma membrane (annexin-V binding). These early events were followed by caspase activation, DNA laddering and loss of plasma membrane integrity (PARP-cleavage, agarose gels, and 7AAD labeling of nuclei). Overexpression of anti-apoptotic bcl-2 in untreated MDA231 cells was associated with a suppression of MCC activity. Further studies showed bcl-2 overexpression eliminated the calcium-activation of MCC and, hence, suggest a mechanism of action for bcl-2. These findings support a role for opening of the MCC early in the apoptotic cascade. Supported by NATO CRG970210 to MLC, ES and KWK; DGICYT grant PB95-0456 to MLC; and NSF grant MCB9513439 to KWK.

Poster 5

Bcl-2 prevents mitochondrial permeability transition due to increased mitochondrial reducing power

Alicia J. Kowaltowski(1,2), Anibal E. Vercesi(2) and Gary Fiskum(1)
(1)Department of Anesthesiology, The University of Maryland Baltimore, MSTF 5-34, 685 West Baltimore Street, Baltimore, MD 21201U.S.A. [Phone]: +01 (410-706-2990 [FAX]: +01 (410-706-2550
(2)Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, SP, Brazil.

Mitochondrial permeability transition (MPT) is a Ca2+-dependent, cyclosporin A-sensitive, nonspecific inner mitochondrial membrane permeabilization associated with membrane protein thiol oxidation [1]. This phenomenon is implicated in the pathogenesis of both apoptotic and necrotic cell death. Bcl-2 is a potent inhibitor of apoptotic cell death that affects mitochondrial activities related to MPT [2]. In this report, we compare the inducibility of the MPT for Bcl-2(+) and (-) PC12 and GT1-7 neural cell lines. Digitonin-permeabilized Bcl-2(-) cells respiring on malate and glutamate exhibited a cyclosporin A-sensitive decrease in mitochondrial membrane potential induced by Ca2+ alone or Ca2+ in the presence of MPT inducers t-butyl hydroperoxide (t-bOOH, a pyridine nucleotide oxidant, 200 mM) or the dithiol reagent phenylarsine oxide (PhAsO, 15 mM). However, Bcl-2(+) cells underwent a decrease in mitochondrial membrane potential only in the presence of PhAsO. In all cases, the drop in mitochondrial membrane potential was accompanied by the release of the pro-apoptotic factor cytochrome c to the extracellular suspension. Also, Bcl-2 overexpressing cells maintained a higher level of reduced pyridine nucleotides when treated with Ca2+ and/or t-bOOH. When the level of reduced pyridine nucleotides was maintained high in Bcl-2(-) cells, in the presence of malate, glutamate, rotenone and succinate, these cells reacted in a manner similar to Bcl-2(+) cells, and exhibited the MPT only when treated with PhAsO. Moreover, Bcl-2(+) cells could undergo MPT at concentration of t-bOOH (800 mM) sufficient to oxidize the elevated levels of reduced pyridine nucleotides. We conclude that the increased reducing power of Bcl-2(+) cells, and not the presence of the Bcl-2 protein itself, is responsible for the resistance to MPT under conditions of oxidative stress induced by Ca2+ alone or Ca2+ plus t-bOOH. Since cytochrome c release from mitochondria under these conditions is related to MPT, the larger reducing power in Bcl-2(+) cells may explain their resistance to cell death (Supported by NS34152 and FAPESP).

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Poster 6

MITOCHONDIAL DNA DAMAGE: DEVELOPMENT OF A QUANTITATIVE TAQMAN-PCR ASSAY FOR LARGE DELETIONS.

A.E. Director[a], C.J.C. Hamel[a†], R. S. Lofts[a†], W.K. Pogozelski[b], P.G.S. Prasanna[a], and W.F. Blakely[a].

[a]Armed Forces Radiobiology Research Institute, Bethesda, MD 20889 and [b]State University of New York at Geneseo, Geneseo, NY 14454.

A specific deletion of 4,977-bp in length is found in human mitochondrial DNA (mtDNA) and is known as the "common" deletion because of its occurrence in association with many genetic diseases as well as its accumulation in “normal” individuals as a function of age[1, 2]. Recently, Kubota *et al*. demonstrated that ionizing radiation induces this deletion in a dose-dependent fashion[3]. We hypothesize that this mtDNA deletion is a persistent biomarker for radiation exposure[4]. A rapid method for quantification of this deletion is essential for biodosimetry applications. Our interest in this deletion as biodosimetric tool has led us to begin developing a fluorogenic 5' nuclease (Taqman) PCR protocol that will substantially improve the quantitative precision and accuracy of measuring wild-type (WT) to deleted mtDNA ratios, reduce analysis time, and allow high sample throughput. Total human lymphocyte DNA was used as a template to amplify fragments representative of WT and deleted mtDNA using the method of Cooper *et al*[2]. Calibration standards are being developed using the cloned sequences of PCR products. Taqman detection of the WT mtDNA calibration standard using standard PCR conditions and a 25-mer WT-specific fluorescent Taqman probe, labeled with the reporter dye 6-carboxyfluorescein (FAM) and the quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA) yielded positive, quantitative results over a broad dynamic range. Development of a calibration standard for the "common" deletion is in progress. This will permit the use of internal calibration curves in measuring the ratio of deleted to WT mtDNA, and aid in determining the feasibility of detection of both WT and deleted mtDNA in a multiplex-PCR platform. We expect this enhanced analytical approach will allow an effective and thorough examination of mtDNA deletions for their potential as accurate, easily measured biomarkers of radiation exposure. (This research was supported by the Armed Forces Radiobiology Research Institute, under work unit number AFRRI-98-3.) [1]Wallace, Sci. Am. 1997, 277, 40. [2]Cooper *et al*., J. Neurol. Sci. 1992, 113, 91. [3]Kubota *et al*., Radiat. Res., 1997, 148, 395. [4]Pogozelski *et al*., Prog. Abs. 44[th] Ann. Mtg. Rad. Res. Soc., 1996, P09-166.
Note. This work was originally presented both at the Environmental Mutagen Society Meeting in Los Angeles, CA, 21 – 26 March ’98 and at the Radiation Research Society Meeting, Louisville, KY, 25– 29 April ’98.

Poster 7

A POLYMORPHISM ELIMINATING THE STOP CODON OF ATP SYNTHETASE SUBUNIT 6 GENE DOES NOT IMPAIR THE FUNCTION OF MITOCHONDRIAL DNA-ENCODED ATP SYNTHETASE SUBUNIT 6.

K. C. Hoffbuhr1,3*, E. Davidson3, B.A. Filiano2, N.G. Kennaway1, M.P. King3

1Dept. of Molecular and Medical Genetics, Oregon Health Sciences University. 2Dept. of Neurology, Columbia University. 3Dept. of Biochemistry and Molecular Pharmacology, Thomas Jefferson University. 233 S. 10th St., Bluemle Rm 308, Philadelphia, PA 19107 (bkcollins@earthlink.net, 215-503-4840, [FAX]: 215-503-5393)

We recently described the identification of a 15bp deletion within the mtDNA-encoded subunit III of cytochrome c oxidase (COX) in a patient with isolated COX deficiency (Keightley et. al, 1996). Sequencing of the patient's mtDNA revealed the presence of a novel polymorphism, a T9205C mutation in ATP synthetase subunit 6 (A6), which changed the A6 stop codon to a glutamine and was present in the mtDNA of both the patient and her unaffected mother. To determine if the effects of the COX III mutation were exacerbated by the presence of the A6 mutation, both wild-type and transmitochondrial cell lines harboring the T9205C mutation were characterized.
The A6 polypeptide in cell lines with the T9205C mutation displayed a slower electrophoretic mobility in SDS-tricine gels compared to wild type. Because the A6 stop codon is eliminated by

the T9205C mutation, this aberrant A6 polypeptide could be produced from either the translation of the polyadenylated A8/A6 mRNA transcript or the translation of a partially processed mRNA transcript encompassing the A8/A6 + COX III mRNA sequences. Two-dimensional electrophoresis indicated that this aberrant A6 translation product was probably derived from the partially processed A8/A6 + COX III mRNA transcript resulting in a full length A6 polypeptide with ten additional amino acids at the carboxyl terminus. The synthesis of this aberrant A6[COX III] translation product, however, had no detectable consequences for rates of ATP synthesis in cell lines with the T9205C mutation compared to wild-type. This study confirms that the T9205C mutation did not contribute to the defect in overall respiratory function observed in transmitochondrial cell lines with the COX III mutation.

Poster 8

Identification of 5'-deoxyribose phosphate lyase activity in human DNA polymerase gamma and its role in mitochondrial base excision repair in vitro

Matthew J. Longley, Rajendra Prasad, Deepak K. Srivastava, Samuel H. Wilson, and William C. Copeland

Laboratory of Molecular Genetics and Laboratory of Structural Biology,
National Institute of Environmental Health Sciences (NIEHS), P.O. Box 12233, Research Triangle Park, NC 27709 (longley@niehs.nih.gov, 919-541-7787, [FAX]: (919-541-7613)

Human mitochondria possess an essential genomic element encoding components required for oxidative phosphorylation, and maintaining the integrity of the mitochondrial genome is essential to prevent debilitating human diseases stemming from mitochondrial dysfunction. The overall mutation rate for mitochondrial DNA is determined by the accuracy of mitochondrial DNA replication, the rate of DNA damage in the oxidative environment of the mitochondrial matrix, and the existence of DNA repair mechanisms. As the only DNA polymerase present in mitochondria, DNA polymerase gamma is necessarily involved both in replication and repair processes. To facilitate our understanding of mitochondrial DNA replication, repair and mutagenesis, the gene for the human DNA polymerase gamma catalytic subunit was overexpressed in recombinant baculovirus infected insect cells, and the 136,000 Da protein was purified to homogeneity. We tested the ability of the pol gamma catalytic subunit to participate in uracil-provoked base excision repair reconstituted in vitro with purified components. Subsequent to actions of uracil-DNA glycosylase and AP endonuclease, human DNA polymerase gamma was able to fill a single nucleotide gap in the presence of a 5' terminal deoxyribose phosphate (dRP) flap. Recombinant human DNA polymerase gamma catalyzed release of the dRP residue from incised apurinic/apyrimidinic sites to produce a substrate for DNA ligase. This dRP lyase activity does not require divalent metal ions, and the ability to trap covalent enzyme-DNA complexes with $NaBH_4$ strongly implicates a Schiff base intermediate in a beta-elimination reaction mechanism. Representing the first evidence that a replicative eukaryotic DNA polymerase possesses dRP lyase activity, this work uniquely assigns two primary roles to DNA polymerase gamma: replication of the mitochondrial genome and DNA synthesis during base excision repair. Therefore, DNA polymerase gamma is vital to maintaining the integrity of both replicating and non-replicating mitochondrial genomes.

Poster 9

REGULATION OF MITOCHONDRIAL GENE EXPRESSION DURING GLUTAMATE EXCITOTOXICITY IN RAT CEREBELLAR NEURONAL CULTURES

KRISH CHANDRASEKARAN, ZARA MEHRABYAN, STANLEY I. RAPOPORT* AND GARY FISKUM
DEPT. OF ANESTHESIOLOGY, UNIV. OF MARYLAND, BALTIMORE; MSTF 5-34; 685 WEST BATIMORE STREET; BALTIMORE, MD 21201(KCHANDRA@ANESTHLAB.AB.UMD.EDU,
410-706-3418, [FAX]: 410-706-2550), * LAB. NEUROSCIENCES, NIA, NIH, BETHESDA, MD 20892
Mechanisms regulating mitochondrial gene expression are not well understood although altered expression likely contributes to the pathophysiology of neurodegenerative diseases. Previously, we have observed that elevation of intracellular sodium ([Na]i) markedly decreases levels of mitochondrial mRNA in cultured neurons (Chandrasekaran et al. Neurosci Abs. 637.2, 1997). This was unexpected since high [Na]i increases energy demand which normally up-regulates mitochondrial gene expression. We hypothesized that high [Na]i following exposure of neurons to excitotoxic levels of glutamate inhibits mitochondrial gene expression, affects their electron transport-dependent activities (e.g., [Ca 2+]i buffering, and free radical generation) and increases their vulnerability to both necrotic and apoptotic cell death. We evaluated this hypothesis by measuring the levels of mitochondrial mRNA, respective protein subunits and enzyme activity [i.e., cytochrome oxidase (C.O) activity] in primary rat cerebellar cultures after a transient exposure to glutamate. Addition of 100 micro molar glutamate + 10 micro molar glycine for 30 min followed by incubation in normal growth medium, resulted in a ~50% reduction in mtDNA-encoded C.O subunit III (C.O III) mRNA in the absence of acute cell death. Experiments are in progress to determine the temporal relationship between reduction in mtRNA, levels of protein subunits, enzyme activity and cell death. Supported by NS34152 and a grant from the IPSEN Institut Henri-Beaufour.

Poster 10

Regulation of mitochondrial gene expression in differentiated PC12 cells

Li-Ing Liu, Hatanpää K., Rapoport S.I. and Chandrasekaran K
Lab of Neurosciences, National Institute on Aging, NIH, Bethesda, MD 20892
(liingliu@helix.nih.gov, 301-594-7753, 301-402-0074)

In Alzheimer's disease (AD) brain, levels of mitochondrial DNA (mtDNA)-encoded genes of oxidative phosphorylation system (OXPHOS), such as cytochrome oxidase subunit I-III (COX I-III), are decreased in vulnerable brain regions, whereas there are no changes in mtDNA-encoded ribosomal RNA and mtDNA. These results suggest regulation occurring at the level of mtDNA transcription or/and turnover of mRNA. We propose that ions may play a role in these regulatory mechanisms, because ATP in the brain is used primarily for ionic pumping during repolarization. To examine the mechanism(s), we developed a cell culture system in which levels of mtDNA-encoded mRNA can be reduced by inhibiting ionic pumping. After differentiated PC12 cells were treated with drugs for various time, total RNA was isolated and levels of COX III mRNA were measured (normalized to ß-actin mRNA). Ouabain (1 mM), a Na+/K+-ATPase inhibitor, caused a 50% decrease in COX III mRNA within 6 h. Addition of monensin (0.1 µM), a sodium ionophore, almost totally abolished COX III mRNA within 6 h. The decreases in COX III mRNA were observed in the absence of significant changes in the levels of mtDNA-encoded ribosomal RNA (12S rRNA) and mtDNA. Measurement of the ADP/ATP ratio in ouabain and monensin treated cells showed that the decrease in COX III mRNA was unrelated to the energetic status of the cell. These data indicate that intracellular sodium is involved in the regulation of mtDNA gene expression. Preliminary data suggest that the effects of elevated sodium on COX III mRNA levels are not dependent on extracellular calcium. The roles of calcium and turnover of COX III mRNA are being studied to further understand mt DNA gene expression.

Poster 11

Two novel heteroplasmic mtDNA mutations associated with severe and complicated clinical presentation by TTGE analysis

Tian-Jian Chen[1], Andrea Gropman[2], and Lee-Jun Wong[1]

[1]Institute for Molecular and Human Genetics, Georgetown University Medical Center, 3800 Reservoir Road, M4000 Main Hospital Building, Washington, DC20007 (chent@medlib.georgetown.edu, 202-784-3094, [FAX]: 202-784-1770)
[2]Institute for Molecular and Human Genetics, Georgetown University Medical Center and Medical Genetics Branch, NHGRI, NIH

Mitochondrial disorders represent a group of heterogeneous diseases which can be manifested in various tissues, such as nerve, muscle, heart, endocrine glands, liver, and kidney. Diagnosis of a mtDNA disorder is difficult due to the board range of clinical expression and the lack of reliable biochemical and pathological markers. Confirmatory diagnosis of a mtDNA disorder is achieved by mutational analysis of mtDNA. We have recently demonstrated that Temporal Temperature Gradient Electrophoresis (TTGE) is a sensitive and effective method to scan mtDNA mutations. Here we report two novel heteroplasmic mutations detected by TTGE. Both mutations are associated with severe and complicated clinical presentation.
The first mutation was found in a 21-month-old boy. He has a remarkable history of failure to thrive, breath holding spell with loss of consciousness. Other clinical findings include encephalopathy, mild dysmorphic features, choleithiasis, bilateral sensorineural hearing loss, liver failure, and moderate global developmental delay. Mutation analysis of mtDNA with TTGE revealed a heteroplasmic mutation, which subsequently confirmed by DNA sequencing to be a novel G5821A mutation located at amino acid carrier arm of the tRNAcys.
The proband of the second mutation is the first born of premature twin males who experienced early respiratory failure and significant lactic acidosis which persisted despite bicarbonate infusion and thiamine supplementation. He died at day 10 of life, and his twin brother died at day 2 of life. Family history was remarkable for a maternal half sibling with short stature and the other maternal half sibling died at 2 months after receiving a DPT vaccination. TTGE analysis revealed a heteroplasmic mutation which was shown by DNA sequencing to be a two base pair (TA) deletion at position 8042 (mt8042 Del TA). This frame shift mutation result in a premature stop codon in cytochrome C oxidase II gene. The mutant gene produces a truncated protein that is 64 amino acids shorter than the wide type protein.
In summary, clinical features of these cases are consistent with the spectrum of findings in mitochondrial cytopathies. One of the novel mutation disrupts the structure of a tRNA, while the other produces a truncated protein. Neither of them was found in more than 200 controls. Thus, the pathogenicity for this two mutations is suggested.

Poster 12

Maternal inheritance in pseudoexfoliation syndrome: a new observation

K. F. Damji;1 E. Stefansson;2 M.Loftsdottir;2 T. Sverrisson;2 E. Thorgeirsson;2 F. Jonasson;2 M. Gottfredsdottir;3 H. S. Bains;1 R. R. Allingham4

1) Dept. of Ophthalmology, University of Ottawa, University of Ottawa Eye Institute, 501 Smyth Rd. Ottawa, Ontario, Canada, K1H 8L6 (kdamji@ogh.on.ca, 613-737-8218, [FAX]: 613-737-8836).
2) Dept. of Ophthalmology, University of Iceland, Reykjavik, Iceland; 3) Dept. of Ophthalmology, University of Michigan, Ann Arbor, MI; 4) Dept. of Ophthalmology, Duke University, Durham, NC.

Background: Pseudoexfoliation (PEX) syndrome is the most common identifiable cause of open angle glaucoma worldwide.
PEX is a late-onset disorder characterized by deposits of fibrillar amyloid-like material within the eye and other organs. Onset is usually in the sixth to seventh decade of life making it difficult to establish the mode of transmission. Purpose: To identify and describe 2 generation families with PEX.
Methods: Probands with PEX syndrome (i.e. PEX but no glaucoma) and those with PEX associated glaucoma were identified through University based eye clinics in Reykjavik and Ottawa. Large families with a history of 2 or more members with PEX were identified. All available members of candidate families over age 45 received a complete eye exam and were ascertained for the presence of PEX and glaucoma.
Results: Six Icelandic and 7 Canadian pedigrees were identified with PEX (with or without glaucoma) in 2 generations. In all cases, transmission appeared
to be matrilineal. Male to offspring transmission (where the mother was unaffected) was not seen in these kindreds. Maternal transmission raises the possibilities of mitochondrial, X-linked, and autosomal inheritance with genomic imprinting. It is intriguing that PEX is consistent with several other criteria for mitochondrial disorders: variable phenotype both within and amongst individuals in the same family, late age of onset, multisystem involvement, and high prevalence in some populations. Mendelian inheritance cannot be excluded and deserves further study.
Conclusion: PEX may be maternally inherited. Further studies are needed to clarify the mode of inheritance and the role of mitochondrial and nuclear DNA in the pathogenesis of this disorder.
Acknowledgements: Green Shield Canada (KFD, Research to Prevent Blindness(RRA), and Barkhauser Glaucoma Research Fund (RRA and KFD.

Poster 13

EVIDENCE FOR MITOCHONDRIAL DYSFUNCTION IN TOXIC AND NUTRITIONAL RETINOPATHIES AND OPTIC NEUROPATHIES

Janis T. Eells, Marina T. Seme and Michele M. Henry

Department of Pharmacology and Toxicology, Medical College of Wisconsin, 8701 Watertown Plank Rd, Milwaukee, Wisconsin 53226
(jeells@mcw.edu 414-456-8632, [FAX]: 414-456-6545)

The clinical features of methanol intoxication are remarkably similar to those of Leber's hereditary optic neuropathy (LHON), nutritional amblyopia, and the recent Cuban epidemic of optic neuropathy (CEON) (Sadun et al., 1994; Rizzo, 1995). We hypothesize that a common pathophysiological mechanism involving impaired mitochondrial function contributes to the retinal and optic nerve dysfunction characteristic of each disease or syndrome. In LHON the defect has been associated with mitochondrial mutations affecting subunits of complex I of the mitochondrial respiratory chain culminating in acute bilateral central vision loss. In both nutritional amblyopia and in the Cuban epidemic of optic neuropathy, the combination of folate and vitamin B12 deficiencies may result in increased endogenous formic acid concentrations due to the disruption of one carbon metabolism (Eells et al, 1996). Formic acid is the toxic metabolite responsible for the visual impairment, blindness and CNS toxicity associated with methanol poisoning. Formic acid has been hypothesized to disrupt visual function by inhibition of the mitochondrial enzyme cytochrome oxidase resulting in a reduction of oxidative phosphorylation essential for

maintenance of neuronal function. To test this hypothesis, we measured cytochrome oxidase (C.O.) activity and ATP concentrations in retina, brain, heart, kidney and liver in a rodent model of methanol intoxication developed in our laboratory. Male-Long Evans rats were intoxicated with methanol as described by Eells (1991). Blood gas determinations, blood formate analyses and ERG recordings were obtained prior to methanol administration and at 12-24 hour intervals following methanol. At 60 hrs animals were euthanized. In one series of studies the posterior retina was processed for C.O. histochemistry. In a second series of studies, C.O. activity and ATP concentrations were measured in tissue extracts prepared from retina, cortex, heart, kidne! y and liver. Methanol-intoxicated rats developed formic acidemia, metabolic acidosis and retinal dysfunction manifested as reductions in the electroretinogram. Morphologic studies coupled with C.O. histochemistry revealed mitochondrial disruption and a profound reduction in retinal C.O. activity in methanol-intoxicated rats compared to control animals. Biochemical measurements also showed a significant reduction in retinal and brain C.O activity and ATP concentrations in methanol-intoxicated rats relative to control animals. Surprisingly, no differences from control values were observed in cardiac, renal or hepatic cytochrome oxidase activity or ATP concentrations in methanol intoxicated rats. The reduction in retinal function, inhibition of retinal and brain C.O. activity, depletion of retinal and brain ATP and retinal mitochondrial disruption produced in methanol-intoxicated rats are consistent with the hypothesis that formate acts as a mitochondrial toxin with selectivity for the retina, optic nerve and brain. Understanding the mechanism of formate-induced mitochondrial dysfunction may not only improve our understanding of the pathogenesis of methanol intoxication, but may also provide valuable insight into the pathogenesis of other acquired and genetic retinal and optic nerve diseases.

Poster 14

Pediatric dystonia due to mtDNA mutation at np 14459--report of the fourth family

Andrea Gropman 1,2, Tian Jian Chen 3, Nicole Sands 3, Philip Pearl 2, Cynthia Tifft 2, and Lee Jun Wong 3

1 NHGRI/NIH, 10 Center Drive, room 10C101, Bethesda, MD 208922 (agropman@nhgri.nih.gov, 301-402-8255, [FAX]: 301-496-7157), 2 Children's National Medical Center, Washington, D.C., 3 Institute for Molecular and Human Genetics, Georgetown University Medical Center, Washington, D.C.

Leber hereditary optic neuropathy (LHON) and pediatric onset dystonia is associated with a heteroplasmic G to A transition at nucleotide position 14459, within the mtDNA encoded ND6 gene. Clinically, this mutation has been reported in only three previous families presenting with LHON alone, LHON plus dystonia, or pediatric dystonia (typical age of onset less than five years). The mutation changes a moderately conserved alanine to a valine at amino acid 72 which is within the most evolutionarily conserved region of the ND6 protein. Pediatric onset disease is associated with evidence of basal ganglia dysfunction, corticospinal tract features and encephalopathy. We report a three year old girl with anarthria, dystonia, spasticity and mild encephalopathy due to heteroplasmic G to A transition at np 14459. In addition, MRI of the brain demonstrated basal ganglia lucencies associated with cerebral and systemic lactic acidosis. DNA analysis of muscle confirmed the mutation and was not seen in over 200 control patients. While the exact number of patients with dystonia who harbor mtDNA mutations is not known, this case demonstrates the relevance of screening for mtDNA mutations in this population. Further molecular studies of the other family members are currently underway.

Poster 15

Genetic Markers of Aging Are Not Detectably Altered by Immunosuppression and/or Infection in Mice.

GL McLaughlin, SZ Shapiro, and JM Pascale

Indiana University School of Medicine, Food and Drug Administration, and University of Panama School of Medicine

Telomere lengths and mitochondrial mutations, especially the common 5 Kb deletion, are considered to be near-universal genetic markers for, and possible contributing factors to, the aging process. The purpose of this study was to examine whether immunosuppression or infections alter telomere lengths and 5 Kb deletion frequencies of mice, HIV-infected or healthy Macques, and within and due to eukaryotic lung pathogens such as Pneumocystis carinii and Histoplasma capsulatum. Telomere length assays were adapted from Geron for humans, primates and rodents using Southern blot and PCR methods. Quantification of 5 Kb deletions was achieved using specific quantitative mimic PCR assays for rodents and humans. Immunosuppression of mice was achieved either by the specific removal of over 95% of CD4+ immune cells as assessed by flow cytometry (using IP injections of anti-CD4 (LT34) antibodies); or by feeding dexamethosone and tetracycline ad lib in water. Control groups incuded normal and Septra-treated mice. In the DNA isolated from healthy control groups and from immunosuppressed and/or infected mouse tissues (lung, kidney, spleen and peripheral blood DNA), as well as normal and HIV-infected macques (peripheral blood DNA), neither average telomere length nor frequencies of 5 Kb deletions were detectably altered. Neither nuclear nor mitochondrial genomes of hosts or eukaryotic pathogens appeared to detectably change due to immunosuppression, infection, or chemotherapy. Markers also did not detectably change in size or frequency after spleen lymphocyte proliferation induced by pathogen or lectin exposures. Similar telomere length and mitochondrial deletion assays using DNA isolated from archived and fresh human tissues, did reveal detectable alterations in both average telomere length and 5 Kb deletions, between groups of 70+, 40-50, and 2-10-year old specimens. Alterations also appeared between assorted individual-matched organ tissue and associated malignant tissues, from prostate, kidney, lung, and CNS samples. Neither immunosuppression nor lung fungal infections in mice, nor HIV infection in Macques, detectably altered these genetic markers within the time frame and conditions of these experiments. Telomeres seemed longer in rodents and primates than in humans, and animals may not have had sufficient range of ages or well enough controlled infectious histories. The use of defined subsets of cells (e.g. CD8+ cells), more closely matched tissues, and more sensitive and species-specific assays for these genetic markers, may be necessary to define contributions of chronic or acute infections to genetic alterations associated with aging.

Poster 16

Oligomycin-driven selection of normal mtDNAs in human cells harboring a pathogenic mutation in the ATPase 6 gene

Giovanni Manfredi, Nihaar Gupta, Antonella Spinazzola, Martel E. Vazquez-Memije, James E. Sadlock, Darryl C. De Vivo, and Eric A. Schon

Columbia University College of Physicians & Surgeons, Dept. of Neurology - Room 4-431
630 West 168th Street, New York, NY, 10032 (eas3@columbia.edu, 212-305-1665, [FAX]: 212-305-3986)

A T->G mutation at position 8993 in human mtDNA is associated with the syndrome neuropathy, ataxia, and retinitis pigmentosa (NARP) and with a maternally-inherited form of Leigh's syndrome (MILS). The mutation results in the substitution of an arginine for a leucine at amino acid position 156 in ATPase 6, a component of the F0 portion of the mitochondrial ATP synthetase complex. Fibroblasts harboring high levels of the T8993G mutation have decreased ATP synthetase activity, but these cells do not display any growth defect under standard culture conditions. We noted that resistance to oligomycin is associated with mutations in the ATPase 6 gene in the same transmembrane domain where the T8993G amino acid substitution is located. We therefore created selective culture conditions using galactose (mutant cells grow poorly in this medium) and oligomycin that elicited a pathological phenotype in T8993G cells and that allowed for the rapid selection of wild-type over mutant cells. We then generated cybrids containing heteroplasmic levels of the T8993G mutation, and showed that selection in galactose-oligomycin caused a significant decrease in the proportion of mutant mtDNA in these cells.

Poster 17

Neuronal Mitochondrial Proliferation in Alzheimer Disease: Evidence for a Fundamental Metabolic Abnormality

George Perry†, Keisuke Hirai†§, Akihiko Nunomura†‡, Robert B. Petersen† and Mark A. Smith†*

†Institute of Pathology, Case Western Reserve University, 2085 Adelbert Road, Cleveland, Ohio 44106 (gxp7@po.cwru.edu, http://www.cwru.edu/med/pathology/perry.htm, §Pharmaceutical Research Laboratories I, Pharmaceutical Research Division, Takeda Chemical Industries Ltd. Osaka 532-8686, Japan, and ‡Department of Psychiatry and Neurology, Asahikawa Medical College, Asahikawa 078-8510, Japan

For over a decade, the role of mitochondrial deficiencies in Alzheimer disease (AD) has been debated, but their role in neuronal degeneration has remained unexplained. Nonetheless, oxidative stress and aging, two key aspects of AD, are associated with mitochondrial DNA (mtDNA) alterations including point mutations, deletions and duplications. This study is based on the premise that mitochondrial (mt) proliferation is a hallmark response to metabolic failure that could be used to show whether metabolic abnormalities are significant to neurons. Since one of the most common alterations in human mtDNA is the 4977-bp deletion (i.e., common 5kb deletion), here, using in situ hybridization, we compared the localization of the 5kb deletion in the hippocampus of patients with AD (n=8) and age-matched controls (n_). We also performed cytochrome oxidase immunocytochemistry and enzyme cytochemistry. We found that the 5kb deletion is markedly accumulated in large pyramidal neurons in AD patients, while very weak labeling was found in control brain. Interestingly, mtDNA without deletions are also accumulated, while neurons with neurofibrillary tangles showed reduced mtDNA, both undeleted and deleted. Interestingly, cytochrome oxidase immunoreactivity paralleled the mtDNA increase, however, enzyme activity was reduced in AD. The 5kb deletion overlaps the neurons labeled strongly with two monoclonal antibodies to nucleic acid oxidative damage marked by 8OH-guanosine (8OHG). Suprisingly, we found the increase in mtDNA is inversely correlated with the extent of 8OHG in the same neurons, suggesting that while mitochondria may be responsible for much of the neuronal oxidative damage in AD, oxidative damage actually decreases during the course of the disease due to failure of mitochondria respiration.

Poster 18

A MITOCHONDRIAL DNA STANDARD REFERENCE MATERIAL FOR MEDICAL DIAGNOSTICS, MUTATION DETECTION, AND FORENSIC APPLICATIONS

Barbara C. Levin, Haiyan Cheng and Dennis J. Reeder

Biotechnology Division, Building 222, Room A353, NIST, Gaithersburg, MD 20899
(barbara.levin@nist.gov, 301-975-6682, [FAX]: 301-330-3447, [URL]: http://www.nist.gov)

The National Institute of Standards and Technology (NIST) is developing a human mitochondrial DNA (mtDNA) standard reference material (SRM 2392) to provide quality control in medical diagnostics, forensic identification, mutation detection, and general sequencing. The need for such a standard stems from the unprecedented rate that sequence data for the human genome and other organisms are being generated and being entered into genetic databases without any mechanism in place to determine if such sequences are correct. These databases will be used for human identification and to determine if various individuals are genetic carriers of polymorphisms or mutations associated with disease. Quality control is needed to assure the accuracy of these databases and of future sequencing of unknown DNA. False positive results could lead to forensic misidentifications, loss of medical insurance, false imprisonment or unnecessary surgery to prevent a presumed future illness. False negative results could result in freeing criminals or not giving patients a needed treatment. Human mtDNA SRM 2392 will include extracted DNA from two control templates (CHR and 9947A) and all the information necessary to perform the polymerase chain reaction (PCR), cycle sequencing steps, gel separation and data analysis to determine the control mtDNA sequences and to ascertain whether the results are correct. The SRM also will include cloned DNA from the HV1 region of the CHR cell line. In addition, sequence information on a third human mtDNA template GM03798 will be provided. The sequences of fifty-eight sets of unique primers will be supplied to allow any area or all of the mtDNA (16,569 base pairs) to be amplified and cycle sequenced. Our results have shown that compared to the Anderson sequence (Anderson et al., 1981) was the first to publish the sequence of the human mitochondrial DNA), none of the differences found in these three templates correspond to any of the published mtDNA mutations associated with specific disease states (Wallace et al., 1997). An inter-laboratory evaluation of the amplification, sequencing, and analysis of the data from the CHR template conducted by four laboratories indicated that three laboratories experienced no difficulties in obtaining the correct sequence. One laboratory, however, did experience problems, an indication that such a laboratory could have used this SRM to inform them that they needed to improve their techniques. Corroboration of the SRM results provides quality assurance that any unknown mtDNA is being sequenced correctly and the resulting medical diagnoses or forensic identifications are accurate. This SRM will be available from NIST in the near future.

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Poster 19

HETEROPLASMY RESPONSIBLE FOR DIFFICULTY EXPERIENCED IN SEQUENCING THE HUMAN MITOCHONDRIAL DNA HV1 REGION CONTAINING THE C-STRETCH

***Barbara C. Levin, †Haiyan Cheng, ‡Mitchell Holland, and *Dennis J. Reeder**

*Biotechnology Division, National Institute of Standards and Technology, Building 222, Room A353, Gaithersburg, MD, †GEO-CENTERS, Inc., Newton Centre, MA, ‡Armed Forces DNA Identification Laboratory, Armed Forces Institute of Pathology, Rockville, MD 20899 (barbara.levin@nist.gov, 301-975-6682, 301-330-3447, http://www.nist.gov)

Sequence analysis of mitochondrial DNA (mtDNA) is being used for human identification especially in those cases where nuclear DNA is highly degraded or non-existent. The distinction between individuals is primarily based on the considerable sequence variation found in the two hypervariable regions (HV1 and HV2) located in the non-coding displacement loop (D-loop). PCR amplification and sequencing of the PCR product of these regions have been generally successful and useful for human identification. In some individuals, however, either or both the HV1 and HV2 regions contain long homopolymer stretches of only cytosine © bases (C-Stretch region) which interfere with sequence analysis in the region following the C-Stretch. The objective of this research was to determine the reason for this interference. Effects of changing the DNA polymerase, PCR additives, and other parameters, such as denaturation and annealing temperatures, cycle number, enzyme concentration, dCTP or dNTP concentrations, magnesium concentrations, and adding formamide were examined. In all cases, the ability to sequence beyond the C-Stretch region was not improved. To determine whether the C-Stretch problem arises during the PCR amplification or cycle sequencing, we used capillary electrophoresis (Bio-Rad Laboratories, Inc. Richmond, CA) to analyze some of the amplification products to determine if they contained more than a single DNA product. The results showed that the control DNA (no C-Stretch problem) generated a symmetric peak indicating a single PCR product; whereas, each of the two DNA products with the C-Stretch problem produced a shoulder peak indicating more than one product. These results suggested that the C-Stretch problem may be due to a heteroplasmic mixture of mtDNA's, each containing C-Stretches of different lengths. The HV1 region was amplified by PCR and the PCR product was cloned into the M13mp18 vector which was used to transfect E. coli TG-1 cells. Using the cloned DNA, we were now able to obtain good sequence data from the HV1 region beyond the C-Stretch. The sequence of these clones showed different numbers of C's. In 19 clones, four had 11 C's, twelve had 12 C's, and three had 13 C's. The questions that now arose were (1) Is the heteroplasmy due to errors made during the PCR process or (2) Does heteroplasmy exist in the mitochondrial DNA of the donor cells? To answer question 1, we reamplified the cloned PCR product DNA and found that the C-Stretch sequencing problem reappeared. To answer question 2, we needed a large quantity of cells in order to isolate and directly clone the mtDNA from the cells rather than the PCR product. White blood cells from the original donor were transformed with the Epstein-Barr virus and immortalized as a tissue culture cell line. MtDNA was isolated from 4 x 108 tissue culture cells, cut with the restriction enzymes Sac1 and Kpn1, separated on a gel and the fragment containing the C-Stretch was cloned into the M13mp18 vector which was used to transfect E. coli TG-1 cells as described above. The DNA from 48 plaques was sequenced and 19 plaques were found to contain C-Stretches. Two had 10 C's, six contained 11 C's, nine contained 12 C's and two had 13 C's. We conclude that good sequence data following a C-Stretch can be obtained from cloned DNA. The inability to sequence through this region is due to a pool of heteroplasmic species containing different numbers of cytosine residues in the C-Stretch. We found that this heteroplasmy may be generated by PCR of the cloned DNA, but it is also present in tissue culture grown donor cells that have never had PCR.

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CONTROL REGION HETEROPLASMIC DIFFERENCES BETWEEN FETAL AND ADULT MITOCHONDRIAL DNA

Lois A. Tully

National Institute of Standards and Technology, Building 222, Room A353, Gaithersburg, MD 20899 (lois.tully@nist.gov, 301-975-4275, Fax 301-330-3447, [URL]: www.nist.gov)

Mitochondrial DNA (mtDNA) sequence analysis of the hypervariable control region has been shown to be an effective tool for forensic and evolutionary studies. Heteroplasmy, or the existence of subpopulations of mtDNA genomes within an individual, has been found to occur in the control region of normal humans. However, the frequency of heteroplasmy, as well as the segregation patterns of control region heteroplasmic variants among human tissues, have not been adequately addressed. In order to better understand the occurrence and patterns of heteroplasmy in human tissues, we have implemented a denaturant gradient gel electrophoresis (DGGE) system designed to examine heteroplasmy in the hypervariable region 1 (HV1) portion of the mtDNA control region. DGGE separates DNA molecules based on their sequence, as opposed to their size, and our system was shown to be capable of detecting heteroplasmic variants present at levels as low as 1%. We used this DGGE assay to screen for heteroplasmy in a total of 181 postmortem tissues from 21 adults (ages 19-52) and 22 fetuses (gestational ages 17-21 weeks). The individuals included in this study had no known genetic disorders. Two to six tissues were examined from each individual, including bone, brain, liver, muscle, blood (adults), and hair (adults). When heteroplasmy was detected, the heteroplasmic position was identified by excising the DGGE bands, and eluting, re-amplifying, and sequencing the DNA. Additionally, the DNA from all heteroplasmic tissues was sequenced to examine the success of heteroplasmy detection by direct sequencing of PCR products. HV1 heteroplasmy in one or more tissues was detected in 13 out of 43 individuals, or 30.2% (95% C.L. 16.5 - 43.9). The majority of heteroplasmic variants occurred at very low levels and were not detected by direct sequencing of PCR products. There was a significant difference in the incidence of heteroplasmy in the fetal and adult populations. In the adult population, 11 out of 21 individuals, or 52.4% (95% C.L. 31.0 - 73.8) were heteroplasmic, whereas in the fetal population, only two out of 22, or 9.1% (95% C.L. 0.0 - 21.1) displayed heteroplasmy. In addition to the significant differences in the frequency of heteroplasmy, the patterns of heteroplasmy were also very different. In each heteroplasmic fetus, heteroplasmy was found in every tissue tested, and in similar proportions among the individual's tissues. In contrast to these observations, several patterns of heteroplasmy were found in the heteroplasmic adults. These included heteroplasmy in some tissues but not others, as well as substantial differences in the proportions of heteroplasmic variants among an individual's tissues. This study demonstrated that HV1 heteroplasmy is a common occurrence in tissues from normal adults. Additionally, the discrepant results in the fetal and adult populations suggest that segregation of control region heteroplasmic variants is not limited to developmental stages, and may be an ongoing process throughout life.

Mitochondrial DNA Deletions

Poster 21

ΔmtDNA^{4977}, A TUMOR SUPPRESSOR MUTATION

[1,4]Sergio U. Dani, [1],4Maria Angela C. Dani, [2]Steve Zullo, [3]Benedito M. Rossi, [2]Carl R. Merril, [4]Andrew J.G. Simpson.

[1]Laboratory of Gerontogenetics and Gene Therapy, Department of Genetics, FMRP Medical School, University of São Paulo, Ribeirão Preto, SP, Brazil; [2]Laboratory of Biochemical Genetics, National Institutes of Mental Health, NIH, Bethesda, USA; [3]Department of Pelvic Surgery, A. C. Camargo Cancer Hospital, São Paulo, SP, Brazil; [4]Ludwig Institute for Cancer Research, São Paulo, SP, Brazil E-mail: sudani@rgm.fmrp.usp.br. Work supported by FAPESP, Brazil.

The frequency of detection of mtDNA4977 deletion mutations has been found to be less in tumors than adjacent normal tissues; in this study of tumor and adjacent normal tissues from 63 cancer patients, mtDNA4977 deletion mutations were detected by competitive PCR amplification in 36 (57.1%) tumors and in 52 (82.5%) of the samples of normal epithelia that were adjacent to the tumors. The mtDNA^{4977} deletions were detected in 44.4% of the breast tumors, 52.5% of the colorectal tumors, and 78.6% of the gastric tumors while deletions were detected in 66.7%, 80.0%, and 100% of the adjacent respective normal epithelial tissues. The competitive PCR amplification assay utilized in this study is normalized to beta-globin gene copy number, and is sensitive enough to detect mtDNA^{4977} deletions if as few as 0.0001% of the mitochondrial genomes are deleted in a tissue. In a study comparing the level of mtDNA^{4977} deletions in tumors and their adjacent normal tissues, limiting dilution PCR showed that the proportion of mtDNA^{4977} deletion was at least 10- to 100 fold higher in the respective control normal tissue (Figure 1). Remarkably, in none of the cases of breast, colorectal or gastric cancer did the proportion of the mtDNA^{4977} deletion in tumors exceed that of the respective normal tissue. The low levels of mtDNA^{4977} deletions in tumors are consistent with contamination of tumor tissue with normal tissue, suggesting that tumors are essentially free of the mutation. These data support the hypothesis that the mtDNA^{4977} deletion mutation prevents neoplastic transformation in normal epithelia, thus introducing the concept of a *tumor-suppressor mutation*.

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REDUCED ΔmtDNA^{4977} DETECTION FREQUENCY IN ALZHEIMER DISEASE BRAINS: A CAUSE-EFFECT PARADOX

[1,4]Sergio U. Dani, [1,4]Maria Angela Castro Dani, [2]Steve Zullo, [3]José Eymard H. Pittella, [2]Carl R. Merril, [4]Andrew J. G. Simpson

[1]Laboratory of Gerontogenetics and Gene Therapy, Department of Genetics, FMRP Medical School, University of São Paulo, Ribeirão Preto, SP, Brazil; [2]Laboratory of Biochemical Genetics, National Institutes of Mental Health, NIH, Bethesda, USA; [3]Laboratory of Neuropathology, Department of Pathology, UFMG Medical School, Belo Horizonte, MG, Brazil; [4]Ludwig Institute for Cancer Research, São Paulo, SP, Brazil. E-mail: sudani@rgm.fmrp.usp.br. Work supported by FAPESP, Brazil.

Using a semi-automatic image analysis system and a colloidal silver staining method, we morphometrically quantified neuritic plaques, neurofibrillary tangles and neuronal size in the parahippocampal gyrus in 52 autopsied patients, including 27 non-neuropsychiatric patients (normal aging) and 25 Alzheimer disease patients. Presence of ΔmtDNA^{4977} (a classical marker of age related mutation accumulation) in the parahippocampal gyrus has been determined by PCR amplification of a new 127 bp sequence formed by this deletion. To control for total mtDNA present in each sample, we have amplified another 127 bp fragment from the 12S subunit of rRNA, which is rarely deleted. Frequency, f, of ΔmtDNA^{4977} was significantly reduced in the Alzheimer disease group (3/25, f=0.12) as compared to the normal aging group (10/27, f=0.37) (p=0.03). This reduced ΔmtDNA^{4977} frequency is consistent with the hypothesis that cells that preferencially accumulate ΔmtDNA^{4977} are the same neurons of the large size class, which develop plaques, then die, leaving tangles.

Poster 23

Deletional scanning of mtDNA in individual cells: a significant fraction of myocytes in the aged human heart contains clonally expanded deleted mitochondrial genomes.

Konstantin Khrapko (1), Natalie J. van Orsouw (2), Xiaomin Zhang (1), Jeanne Y. Wei (1), and Jan Vijg (2)

Division on Aging, Harvard Medical School, 77 Avenue Louis Pasteur, Suite 921, Boston, MA, 02115, (khrapko@wccf.mit.edu, 617-667-0973, [FAX]: 617-667-0980)
(1) Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02115
(2) Institute for Drug Development, Cancer Therapy, and Research Center, San Antonio, TX 78229

It has been proposed that accumulation of somatic mitochondrial DNA (mtDNA) mutations is a major causal factor in the aging process. However, this hypothesis is difficult to test. Although mtDNA mutations, especially deletions, increase with age, it is not clear whether such increases are physiologically relevant. To assess their relevance, quantitative information on the cell-to-cell distribution of all possible mtDNA mutations in aged tissues is needed. In the present study, we used PCR amplification of full-length mitochondrial genomes from single cells followed by agarose gel electrophoresis, to scan human cardiomyocytes for all possible large deletions in mtDNA. Analysis of almost 200 individual cells that were derived from two middle-aged and two centenarian donors demonstrate that while most of the cells contain no deletions, in certain cardiomyocytes up to about 1/3 of the mtDNA molecules carried a particular deletion. Each affected cell contained only one type of deletion while different cells contained different deletions, which indicates that all deletions found in a particular cell resulted from a clonal expansion of one mutated mtDNA molecule. These deletion-rich cells were found exclusively in the hearts of centenarians, where they occurred at a frequency of almost 1 in 10 cells. These observations suggest that mitochondrial mutations have the potential to play an important role in human myocardial aging. Our ability to amplify full- length mitochondrial genomes from single cells puts us in the position to extend deletional scanning to the analysis of all possible mtDNA mutations in a single cell. Indeed, once mtDNA from a single cell is amplified, exhaustive scanning for point mutations (in addition to deletions) can be done by two-dimensional electrophoresis [van Orsouw (1998)], or other scanning methods. We further propose target mutational scanning of mtDNA to cells demonstrating certain age-related defects (such as loss of cytochrome C oxidase) to test the hypothesis that such defects are caused by mtDNA mutations.

Reference:
N. J. van Orsouw, X. Zhang, J. Y. Wei, D. R. Johns, J. Vijg. Mutational scanning of mitochondrial DNA by two-dimensional electrophoresis. Genomics 52, 27-36 (1998)

Poster 24

IN SITU PCR ASSAY FOR “COMMON” HUMAN MITOCHONDRIAL DNA DELETION: METHODOLOGICAL AND PRACTICAL APPROACHES

Sergei B. Melnov1, Steven J. Zullo2, Pataje G.S. Prasanna3, Alexandra C. Miller3, Carl R. Merril2, and William F. Blakely3

1Institute of Nuclear Medicine and Endocrinology, Minsk, Belarus (melnov@mx.afrri.usuhs.mil, sbm@melnov.belpak.minsk.by, 301-295-9172, [FAX]: (301-295-6567) 2Laboratory of Biochemical Genetics, National Institute of Mental Health-NIH, Bethesda, MD USA; 3Armed Forces Radiobiology Research Institute, Bethesda, MD USA.
Melnov, Sergei B. Melnov

Recent studies have further characterized different pathologies that are due to mitochondrial mutations. Cytological identification and localization of mitochondrial DNA (mtDNA) mutations using DNA-sequence based methods would promote our understanding of the genesis of such diseases and provide useful diagnostic tools. We have developed an in situ PCR method involving a “common” 4977-bp deletion in the human mitochondrial genome (mtDNA4977). This deletion has been associated with diseases including Pearson’s syndrome, Kearns-Sayre syndrome, ophthalmoplegia, cardiomyopathy, as well as aging and exposure to ionizing radiation.

Chronic exposure to free radicals created by electron-transport chain activity, absence of sophisticated repair mechanisms, and frequent mtDNA replication may also contribute to an accumulation of mutations in the mitochondrial genome. We utilized established human lymphocyte cell lines, including one that maintains the mtDNA4977 deletion at around 50% of the total mtDNA. Primer sets were selected to detect this deletion, as well as total mtDNA by assaying a mitochondrial ribosomal RNA gene. Each primer set was labeled with a distinctive fluorochrome, which in a multiplex application permitted cytological confirmation of their co-localization. The coincident localization of total and deleted mtDNA genomes with mitochondrial organelles was corroborated by the combined use of the in situ PCR assays with a mitochondrial membrane probe. Quantitative analyses were also performed using immunoenzymatic based detection methodology to detect cells containing deleted mtDNA in Giemsa-counterstained preparations. We have applied this assay to other cell model systems including peripheral blood lymphocytes, leukemia (Molt-4) cell line, and an immortalized osteoblastic (HOS) cell line and demonstrated that the expression of this deletion varied significantly.
This research was supported by the Armed Forces Radiobiology Research Institute, under work unit number AFRRI-98-3, and by a training fellowship from the International Atomic Energy Agency.

Poster 25

Analysis in tissue culture of rearranged mtDNAs associated with Kearns-Sayre syndrome

Yingying Tang, Eric A. Schon, Ekkehard Wilichowski*, Martel E. Vazquez-Memije, Edgar Davidson, and Michael P. King

Columbia University College of Physicians & Surgeons; *Universitäts-Kinderklinik, Abteilung Pädiatrie/Neuropädiatrie, Göttingen, Germany

We have analyzed transmitochondrial hybrid cells containing large-scale rearrangements (both a partial duplication and a partial deletion) of human mtDNA. Mitochondria from a patient with Kearns-Sayre syndrome who harbored these rearrangements were fused with human cells lacking endogenous mtDNA, and cybrids containing 100% wild-type mtDNA, 100% duplicated mtDNA (dup-mtDNA), and 100% deleted mtDNA (del-mtDNA) were isolated and examined for their their genetic and biochemical characteristics. In contrast to the 100% del-mtDNA lines, there were no obvious respiratory chain or protein synthesis defects in the 100% dup-mtDNA lines. Surprisingly, the number of mtDNA molecules per cell in the 100% duplication and 100% deletion lines was inversely proportional to the size of the respective genomes, indicating that cells maintain a constant mtDNA mass, not a constant copy number, of mitochondrial genomes. The steady-state level of mtDNA transcription was also altered in the rearranged lines, suggesting that mtDNA expression is regulated post-transcriptionally.

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MITOCHONDRIAL INTERMEDIATE PEPTIDASE AFFECTS IRON HOMEOSTASIS IN YEAST MITOCHONDRIA

Grazia Isaya & Steven S. Branda

Mayo Clinic and Foundation, 200First Street SW, Hilton Building 210F, Rochester, MN 55905 (isaya@mayo.edu, 507-266-0110, [FAX]: 507-284-1399)

We have found a functional interaction between the yeast frataxin homologue (Yfh1p, polypeptide; YFH1, gene) and the yeast mitochondrial intermediate peptidase (YMIP, polypeptide; MIP1, gene). YMIP is a metallopeptidase localized to the mitochondrial matrix, which catalyzes the second of two processing steps required for the maturation of a number of imported mitochondrial proteins. YMIP substrates are initially synthesized as larger precursors containing the motif Rx( )(F/L/I)xx(S/T/G)xxxx( ) at the C-terminus of their leader peptide. These precursors follow the general mitochondrial protein import pathway, and are first cleaved by the mitochondrial processing peptidase (MPP) at position -2 from the R residue in the motif. This initial cleavage yields a processing intermediate containing a typical N-terminal octapeptide, (F/L/I)xx(S/T/G)xxxx, which is specifically removed by YMIP to form the mature protein. YMIP substrates include proteins involved in electron transport, mitochondrial DNA (mtDNA) maintenance, and heme biosynthesis, and consequently chromosomal disruption of MIP1 (mip1) leads to loss of respiratory function. The human MIP is also primarily responsible for the maturation of proteins involved in respiration, and can complement loss of YMIP activity in S. cerevisiae, suggesting that the role of this leader peptidase is conserved in eukaryotes.
YFH1 was originally isolated as a high-copy suppressor of a yeast mutant otherwise unable to grow on low-iron media. This and other studies further demonstrated that YFH1 disruption (yfh1) leads to accumulation of iron in the mitochondria, hypersensitivity to oxidative stress, loss of mtDNA integrity, and impaired respiratory function. In light of these results, it was proposed that Yfh1p is normally required for mitochondrial iron homeostasis, and that the elevated iron levels in yfh1 mitochondria may promote formation of reactive oxygen species which damage mtDNA as well as proteins containing iron-sulfur clusters. The presence of iron deposits and multiple iron-sulfur enzyme deficiencies in the myocardium of FRDA patients suggest that a similar mechanism may be responsible for FRDA. We have independently isolated YFH1 based on its ability to rescue the respiratory defect of a temperature-sensitive yeast mip1 mutant (mip1ts). This initial finding has led to genetic and biochemical studies showing a functional interaction between Yfh1p and YMIP.

Poster 27

Structure-function Analysis of Human Mitochondrial DNA Polymerase: Relevance to Antiviral Drug Toxicity.

Susan E. Lim and William C. Copeland.

LMG, MDE3-01, PO Box 12233, 111 T. W. Alexander Dr Research Triangle Park, NC 27709 (lim@niehs.nih.gov, 919-541-5417, [FAX]: (919-541-7613)

The mitochondrial DNA polymerase is the only cellular replicative DNA polymerase that is inhibited by antiviral nucleotide analogs, such as AZT, ddC, ddI, 3TC, and D4T, drugs currently approved against HIV. Thus, understanding the interaction of these antiviral nucleotide analogs with the mitochondrial DNA polymerase is relevant for an effective antiviral drug's regimen. Human mitochondrial polymerase gamma (pol gamma) is low abundance in cells and purification of this enzyme has been a major task. Recently, we cloned, over-expressed and purified the large catalytic subunit of human mitochondrial polymerase in a baculovirus expression system. Based on the protein sequence alignments with other family A polymerases, we identified the conserved regions in exonuclease and polymerase domains. We generated His-tagged pol gamma with mutation at Aspartate at position 198 and Glutamate at residue 200 to Alanines. This double mutant is completely devoid of exonuclease activity (exo--). In motif B of pol gamma the amino acid Y955 is conserved among pol I family and it is showed to interact with the ribose of the incoming nucleotides. Using this exo- background, Y955A and Y955F were expressed with N-terminal histidine tagged in Sf9 insect cell lines and purified to homogeneity. Steady-state studies showed that Y955F had normal poly rA/dT activity with Km of 2mM similar to wild type while Y955A activity was reduced to 20% of wild type with Km of 100mM. Initial studies with ddTTP indicated 50% of Y955F activity was inhibited at 0.1mM. However, Y955A was at least 200 fold resistant to ddTTP. The nature of these mutants with AZT drug analog is being studied. These kinetics data of mutant proteins will provide the insights of how these critical amino acids interact with the incoming nucleotides.

Poster 28

5S rRNA is imported into mammalian mitochondria

Paolo J. Magalhães, Antonio L. Andreu, and Eric A. Schon

Dept. of Neurology, Columbia University - Room 4-431, 630 West 168th Street, New York, NY 10032 (eas3@columbia.edu, 212-305-1665, [FAX]: 212-305-3986) *Universitäts-Kinderklinik, Abteilung Pädiatrie/Neuropädiatrie, Göttingen, Germany

Mammalian mitochondrial ribosomes contain two prokaryotic-like rRNAs, 12S and 16S, both encoded by mitochondrial DNA (mtDNA). As opposed to cytosolic ribosomes, however, these ribosomes are not thought to contain 5S rRNA. For this reason, it has been unclear if 5S rRNA, which can be detected in mitochondrial preparations, is an authentic organellar species imported from the cytosol or is merely a co-purifying cytosol-derived contaminant. We now show that 5S rRNA is tightly associated with highly-purified mitochondrial fractions of human and rat cells, and that 5S rRNA transcripts derived from a synthetic gene transfected transiently into human cells are both expressed in vivo and are present in highly-purified mitochondria and mitoplasts. We conclude that 5S rRNA is imported into mammalian mitochondria, but its function there still remains to be clarified.

Poster 29

PURIFICATION AND CHARACTERIZATION OF A RAT LIVER MITOCHONDRIAL THYMINE GLYCOL ENDONUCLEASE.

R. Stierum, D. Croteau#, V. Bohr.

Laboratory of Molecular Genetics, NIA, NIH, 5600 Nathan Shock Drive Baltimore, MD 21224.
#Currently at: Department of Molecular and Cellular Biology, UC Berkeley, Berkeley, CA 94720.
(StierumR@grc.nia.nih.gov, 410-558-8090, Fax: 410-558-8157)

Mitochondrial (mt) DNA is exposed to damage caused by oxygen radicals produced during oxidative phosphorylation. Structural alterations in mtDNA and associated mt dysfunction have been correlated with aging and diseases. Accumulation of oxidative mtDNA damage may be responsible for these mtDNA alterations. MtDNA repair is therefore important in preventing mt dysfunction. Here, we report the purification of a novel rat liver mt thymine glycol (TG) endonuclease (mtTGendo). We observed that DEAE-fractionated extracts from purified rat liver mt nicked OsO4-treated plasmid DNA. Using a radiolabeled oligonucleotide duplex containing one TG, damage-specific incision at the modified thymine was observed. Utilizing subsequent cation exchange, hydrophobic interaction and size exclusion (SE) chromatography, it was shown that mtTGendo has a KCl optimum of ~75 mM and is EDTA-resistant. Active SE fractions displayed a single band of ~37 kDa on a silverstained gel. Further, mtTGendo has an associated AP-lyase activity. Duplexed oligonucleotides containing 8-oxodeoxyguanosine or uracil and single-stranded oligonucleotide containing one TG are no substrates for mtTGendo. Given the fact that TG blocks DNA replication and is slightly mutagenic, mtTGendo may help to prevent accumulation of structural mtDNA changes, mt dysfunction and associated (patho)physiological alterations in vivo.

Poster 30

A SEARCH FOR GENES AFFECTING THE FIDELITY OF MITOCHONDRIAL DNA REPLICATION AND REPAIR

Micheline Strand, Olivia Dominick, and William Copeland

National Institute of Environmental Health Sciences, Laboratory of Molecular Genetics, NIEHS, P.O. Box 12233, MD E3-01, Research Triangle Park, North Carolina 27709
(Strand@NIEHS.NIH.GOV, 929-541-5424)

In order to find some of the missing components of mitochondrial DNA replication and repair, we have initiated a genetic search in the yeast Saccharomyces cerevisiae. To assay mitochondrial mutation rates we are using a nuclear Arg8 gene, recoded for the mitochondrial genetic code, that has been inserted into the mitochondrial genome. A mutant of this gene was isolated, and mutation rates can be determined by assaying reversion on SD-arg plates. This method differs from the erythromycin assay as it does not require functioning mitochondria and the reversion is a dominant mutation.There are several approaches for isolating new genes. We are using both tagged gene disruptions as well as ethylmethane- sulfonate (EMS). To date we have identified 14 EMS- mutagenized yeast strains and 16 tagged disruptions with an increase in the mitochondrial mutation rate of 5- to 60-fold. Fourteen of the tagged disruptions are in genes with no known function. We have identified the function of one of these unknown genes as regulating copper uptake. Deletion of this gene results in lethality with copper doses as low as 25uM. The mutant also has a dramatically elevated rate of mitochondrial loss. We have determined that deletion of this gene increases mitochondrial mutation rates but has no effect on nuclear mutation rates. Sequence analysis predicts that the protein does not directly bind copper, and probably senses intracellular copper levels via Ace1 binding. We are currently doing competitive PCR to determine the effect of Ace1 and copper on the transcription of this gene. We are also measuring total copper uptake in the wild type and the mutant. We predict that the effect of having a defect in this protein in humans may produce a phenotype similar to that of Wilson's disease.

Poster 31

Cardiomyopathy and cell death in transgenic mice expressing a proof-reading deficient mitochondrial DNA polymerase in the heart

Peter Zassenhaus

Department of Molecular Microbiology and Immunology, Saint Louis University Health Sciences Center, 1402 S. Grand Blvd, Saint Louis, Missouri 63104 (ZASSENP@WPOGATE.SLU.EDU, 314-577-8444, Fax:314-773-3403)

Transgenic mice were created expressing a proofreading deficient mitochondrial DNA polymerase in the heart. The transgene was driven by the cardiac alpha myosin heavy chain promoter which is activated shortly after birth. By Day 3, greater than 95% of all mitochondrial DNA polymerase in both ventricular and atrial tissue was derived from the transgene. By DNA sequencing and PCR analysis of mitochondrial DNA point mutations in mitochondrial DNA were detected as early as Day 7 and their frequency progressively increased with time. At 1 month of age these mice developed a progressive, dilatory cardiomyopathy. Conduction abnormalities were revealed by ECG and sudden death occurred in some animals starting at this age. Histochemical analysis of cardiac tissue revealed sporadic myocytic death, minimal inflammation, and increasing fibrosis. By electron microscopy, however, no significant ultrastructural changes in mitochondria were evident; likewise, analysis of mitochondrial respiratory function, cytochrome spectra, and respiratory enzyme activity showed no significant deficiencies in the transgenic compared to the control heart. Neither mitochondrial DNA or RNA levels differed from controls in transgenic animals, nor was there a detectable increase in oxidative damage to mitochondrial DNA in the transgenic heart. By TUNEL stain, significantly elevated levels of positively-staining myocytes were found in the transgenic heart beginning at about 2-3 weeks of age. The frequency of TUNEL-positive cells remained relatively constant thereafter, despite the increasing level of mitochondrial DNA mutations. Whether TUNEL-positive cells reflect ongoing apoptosis is not confirmed at the moment. These observations suggest that the accumulation of point mutations in mitochondrial DNA may not significantly impair mitochondrial respiratory function or cause high levels of oxidative stress. Rather, high levels of point mutations appear to cause cell death by as yet uncharacterized mechanisms.

Poster submitted but not present

A COMPARISON OF THE ACTIVITIES OF MCC AND PSC, THE PROTEIN TRANSLOCATING CHANNELS OF MITOCHONDRIA

C. Muro[1], M. L. Campo[1], and K.W. Kinnally[2]

[1]Dpto. de Bioquímica, Univ. de Extremadura, Spain; [2]Div. of Molec. Med., Wadsworth Center, Empire State Plaza, Albany, N.Y. and Dept. of Biomed. Sci., Univ. at Albany, Albany, NY.

About 95% of all mitochondrial proteins are encoded in the nucleus and synthesized in the cytosol. These precursor proteins are imported into mitochondria by import complexes called Tim and Tom in the inner and outer membranes, respectively. MCC and PSC are the water-filled channels that putatively function in protein translocation in these Tim and Tom complexes. We have conducted a comparative study of the single channel characteristics of MCC and PSC from *S. cerevisiae* mitochondria reconstituted in proteoliposomes using patch-clamp techniques. We determined the functional effects on MCC and PSC behavior of structurally modifying the Tim and Tom complexes by deleting or proteolytically cleaving components. Tim23p and Tom22p are thought to be the receptors of the Tim and Tom complexes, respectively, that recognize the mitochondrial targeting region of precursor proteins. Importantly, intact Tim23p and Tom22p were requisite for regulation of MCC and PSC by targeting peptides. Loss of Tom70p and Tom20p had no effect on PSC or MCC. These data are consistent with the idea that MCC and PSC are distinct entities whose activities are regulated by targeting peptides and by modifications to the Tim and Tom complexes, respectively, and that MCC and PSC are the protein translocation channels of mitochondria. This work was supported by DGICYT grant PB95-0456 and Junta de Extremadura y Fondo Social Europeo grant EIA94-11 to MLC and NSF grant MCB9513439 to KWK.

Poster submitted but not present

An Arg503Cys substitution in the carnitine palmitoyl transferase 2 gene confers malignant hyperthermia and variable myopathy.

G.D. Vladutiu, R.T. Taggart, D.Smail, H.B. Lindsley*, and K. Hogan.**

Dept. of Pediatrics, State Univ. of NY at Buffalo, Dept. of Medicine, Kansas, University-Kansas City and Dept. of Medicine*, Univ. of Wisconsin-Madison**.

Adult-onset carnitine palmitoyl transferase II (CPT II) deficiency is characterized by muscle pain, stiffness, rhabdomyolysis and myoglobinuria. Symptoms may be triggered by exercise, fasting, and extremes in ambient temperature. Events resembling malignant hyperthermia (MH) (tachycardia, fever, metabolic acidosis, muscle destruction) have been reported in patients with CPT II deficiency undergoing surgery with volatile anesthetics or depolarizing muscle relaxants. We report 2 individuals who are heterozygous for a C to T transition at nucleotide 2023 resulting in a substitution of cysteine for arginine at amino acid residue 503 in a highly conserved region of the CPT2 gene. Both patients were negative when screened for 13 other previously described CPT2 mutations. One patient survived an episode of MH during surgery at 4 years of age (base excess -11.3 mmol/L, CPK >5000 mU/mL). Now 21 yrs old, this patient has never experienced intercurrent myopathic symptoms. Enzymatic analysis of her transformed lymphoblasts demonstrated CPT II activity to be 58% of the normal reference mean. The second patient exhibits progressive weakness and myopathic symptoms worsened after surgery. CPT II activities in this patient's lymphoblasts and muscle biopsy were reduced to 47% and 13% of normal respectively, with decreased muscle OXPHOS enzymes ranging from 22 to 49% of normal. It is uncertain whether the Arg503Cys mutation or as yet undetected mitochondrial defects account for the generalized effect on mitochondrial metabolism specific to muscle. This patient's son also has myopathic symptoms and is heterozygous for the Arg503Cys mutation. Our findings suggest that heterozygosity for the Arg503Cys mutation is sufficient to predispose an individual to variably expressed disease phenotypes in distinct genetic backgrounds. In common with MH-associated mutations in skeletal muscle RYR1 and CACNL1A3 genes, our clinical, biochemical and genetic evidence indicates that in some cases, the Arg503Cys CPT2 substitution may cause a latent myopathy which only becomes apparent after specific anesthetic exposure. Supported by a grant from the Muscular Dystrophy Association.

Poster 32

Mitochondrial transfer and the creation of transmitochondrial mice.

C.A. Pinkert

Department of Comparative Medicine and The UAB Transgenic Animal/ES Cell Resource, The University of Alabama at Birmingham, VH2271670 University Blvd, Birmingham, AL 35244-6732 (pinkert@uab.edu, 205-934-9574, Fax, 205-975-4390, http://transgenics.bhs.uab.edu)

Aberrant metabolic and cellular phenotypes exist due to mutations arising exclusively within the mitochondrial genome. The ability to manipulate the mitochondrial genome and to regulate the expression of mitochondrial genes would provide one possible mode of genetic manipulation and therapy. While gene transfer became commonplace in a host of cell types and organisms, with the exception of cell fusion procedures, transfer of nuclear DNA had been the only demonstrable form of mammalian gene transfer. Until recently, in vivo mitochondria transfer remained a technological hurdle in the development of mitochondria-based gene transfer and genetic therapies. Therefore, the development of technology to establish mitochondria transfer as a viable technique to genetically engineer mouse models was addressed. Initially, such models would serve to explore mitochondrial dynamics in an in vivo system. To date, we have developed a method for mitochondria isolation and interspecific transfer of mitochondria in a mouse model. Mitochondria were isolated from Mus spretus liver samples for microinjection into fertilized ova obtained from superovulated Mus musculus domesticus females. Electron microscopic observations of the mitochondria preparations used for microinjection demonstrated intact mitochondrial vesicles with little microsomal contamination. Species-specific nested PCR primers complementary to sequence differences in the mitochondrial DNA D-loop region were used to detect high rates of successful transfer of foreign mitochondria after isolation and injection into mouse zygotes. The creation of heteroplasmic transmitochondrial mice represents a new model system that will provide a greater understanding of mitochondrial dynamics. Current efforts to establish and further characterize our mitochondrial transfer-derived mouse models will be discussed. Ultimately, the development of therapeutic strategies for human metabolic diseases affected by aberrations in mitochondrial function will be a primary focus of this research.

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Toward a Mitochondrial Chip?

Peter C. Preusch

National Institute of General Medical Sciences, 45 Center Dr, MSC 6200, Bethesda, MD 20892-6200 (preuschp@nigms.nih.gov, 301-594-5938, [FAX]: 301-480-2802)

The development of chip-based technology for DNA and RNA analysis presents new opportunities for research. The NIH is interested in fostering the application of new technology to appropriate problems. The study of mitochondrial hereditary diseases and the spectrum of mitochondrial gene expression in the normal human population would seem to be excellent topics for application of this technology. There may also be basic research problems where studies in model organisms would be facilitated by chip-based methods. The number of relevant genes is tractable and many have been clearly identified. This is certainly so, if one considers only the mitochondrially-encoded genes. A substantial number of the nuclear genes essential to mitochondrial function have also been identified. Thus, it may be possible to define the appropriate set of oligonucleotides that should be represented on a mitochondrial chip that could serve the needs of multiple investigators. This poster will not present the answer to that question, rather, it will provide an opportunity for meeting participants to discuss the issues with NIH staff. Important issues to resolve would include:

What biological problems could be most usefully addressed using chip technology?
What specific genes should be included on a mitochondrial chip?

What improvements in chip manufacturer and chip reader technology are needed?
Who would be the principal users of a mitochondrial chip?
Who would develop and manufacture the chips using what specific technology?
What grant mechanisms would support the development of the chips?
What grant mechanisms would support the most efficient access to the technology?
How much would it cost?
Comments on these issues are most welcome. Please direct your thoughts to the author by Email: preuschp@nigms.nih.gov.